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TITLE:

Generation of Human 3D Lung Tissue Cultures (3D-LTCs) for Disease Modeling

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SHORT ABSTRACT

Here, we present a protocol for the preparation of agarose-filled human precision-cut lung slices from resected patient tissue that are suitable for generating 3D lung tissue cultures to model human lung diseases in biological and biomedical studies.

LONG ABSTRACT

Translation of novel discoveries to human disease is limited by the availability of human tissue-based models of disease. Precision-cut lung slices (PCLS) used as 3D lung tissue cultures (3D-LTCs)

represent an elegant and biologically highly relevant 3D cell culture model, which highly resemble *in situ* tissue due to their complexity, biomechanics and molecular composition. Tissue slicing is widely applied in various animal models. 3D-LTCs derived from human PCLS can be used to analyze responses to novel drugs, which might further help to better understand the mechanisms and functional effects of drugs in human tissue. The preparation of PCLS from surgically resected lung tissue samples of patients, who experienced lung lobectomy, increases the accessibility of diseased and peritumoral tissue. Here, we describe a detailed protocol for the generation of human PCLS from surgically resected soft-elastic patient lung tissue. Agarose was introduced into the bronchoalveolar space of the resectates, thus preserving lung structure and increasing the tissue's stiffness, which is crucial for subsequent slicing. 500 µm thick slices were prepared from the tissue block with a vibratome. Biopsy punches taken from PCLS ensure comparable tissue sample sizes and further increase the amount of tissue samples. The generated lung tissue cultures can be applied in a variety of studies in human lung biology, including the pathophysiology and mechanisms of different diseases, such as fibrotic processes at its best at (sub-)cellular levels. The highest benefit of the 3D-LTC *ex vivo* model is its close representation of the *in situ* human lung in respect of 3D tissue architecture, cell type diversity and lung anatomy as well as the potential for assessment of tissue from individual patients, which is relevant to further develop novel strategies for precision medicine.

INTRODUCTION

Chronic and acute lung diseases are a major cause of morbidity and mortality worldwide[1]. For patients with chronic lung diseases like obstructive pulmonary disease (COPD)[2], severe asthma[3], lung cancer[4] and diffuse parenchymal lung diseases[5], curative therapies are currently not available. Although studies in animal models for lung diseases have deepened the understanding of disease pathomechanisms[6] and have led to the identification of potential novel therapeutic targets[7-9], these models exhibit relevant biological and physiological differences when compared to humans[10]. To overcome these discrepancies between murine and human biology as well as anatomy, human *ex vivo* 3D lung tissue culture (3D-LTC) systems are used in various areas of biomedical research. These 3D-LTC culture systems are based on precision-cut lung slices (PCLS). The generation of PCLS *ex vivo* allows analysis of a third spatial dimensionality, which allows for the investigation of the spatial and functional relationships of cells in entire alveoli and airways[11], as well as the interstitium, vasculature and mesothelium. Notably, PCLS *ex vivo* models are multicellular, meaning that they contain most functional cells of *in situ* lungs, thus closely representing the cells' native biological environment and thus overcoming the limited cell-cell and cell-matrix interaction in most 2D cell culture approaches. Up to now, *ex vivo* murine PCLS were used to model pulmonary diseases, like COPD[12], lung fibrosis[13], lung cancer[14], viral infection[15,16], bronchopulmonary dysplasia[17], and asthma[18]. However, a considerable proportion of novel drug therapies in human lung diseases that were investigated in clinical trials do not translate to the clinic due to their lack of efficacy or safety, assumingly due to yet considerable differences between human and murine biology and disease[19-21].

Over several years, human PCLS have been largely used to assess lung toxicity of chemicals and drugs. Only recently, human lung tissue has been used from patients with COPD[22,23], asthma[24],

and lung fibrosis[25], to pursue pathophysiological and pharmacological studies. By using resected patient organ material and generating PCLS thereof, one can recapitulate major disease hallmarks in a complex 3D tissue environment[22] representing and maintaining most of the native cellular diversity of the organ. Moreover, diseased tissue applied in a variety of experimental setups was shown to mimic disease-like changes in liver, intestine and kidney[26-29].

However, processing of lung tissue remains challenging for several reasons. Unlike solid tissue, native lung parenchyma tends to collapse without ventilation and exhibits lower tissue stiffness. These properties impede the slicing of the tissue. Thus, filling of airways and the alveolar space with low-melting point agarose preserves the native lung structure and provides the stiffness needed for precision-cut slicing of murine and human lungs[30]. Human lung resectates donated for research purposes are by their nature anatomically, genetically and physiologically highly diverse, thus often presenting a high inter-patient variability when performing experiments[25]. In contrast to the whole lobe or whole lung explants, lung samples resected by means of thoracic surgery do not necessarily follow the anatomical segments and, therefore, require special preparation. In this article, we provide a detailed and optimized protocol for the generation of human PCLS from resected lung tissue and their subsequent cultivation and experimental use to model lung disease.

PROTOCOL

The use of human tissue was approved by the ethics committee of the Ludwig-Maximillian University [Munich, Germany (project number 455-12)]. Tumor-free human lung resections were provided by the Asklepios Biobank for Lung Diseases (Gauting, Germany, project number 333-10).

Note: All procedures of human PCLS production (**Figure 1**) are done under a sterile laminar flow hood.

1. Preparation of Instruments and Materials

1.1) Prepare all materials for the inflation of lung tissue with agarose as described below.

1.1.1) Prepare the cultivation medium: Dulbecco's Modified Eagle Medium (DMEM) F-12 supplemented with L-Glutamine, HEPES, 10,000 IE Penicillin, 10,000 IE Streptomycin and 0.1% (v/v) fetal bovine serum.

Note: Medium is used at 37 °C.

1.1.2) Prepare a sterile metal tray covered with tissue paper. Place a sterile 15 cm cell culture dish on the tray.

1.1.3) Fill the cell culture dish with the 15 mL of cultivation medium.

1.1.4) Prepare a 3% (w/v) agarose solution by dissolving the appropriate amount of low-melting point agarose in a minimum of 30 mL of cultivation medium.

1.1.5) Heat the solution in a microwave until boiling. Cool the agarose solution to 42 °C in a water bath. Keep the liquid agarose solution stored in the water bath.

1.1.6) Prepare several 50 mL conical tubes filled with the liquid agarose.

2. Resected Lung Tissue

2.1) Store fresh tumor free lung tissue of lobectomy resectates immediately after resection in DMEM F-12 medium at 4 °C until step 3.

2.3) Do not exceed cold ischemia time of 4-8 hours prior to processing.

3. Inspection and Selection of Resected Tissue Prior to Agarose Filling

3.1) Lift the tissue from the medium with tweezers. In order to avoid any damage to the tissue, especially to the pleura, handle the tissue with tweezers at the airways only.

3.2) Score the tissue quality by criteria of the Lung Agarose Filling Score defined in **Table 1**.

3.3) Proceed to step 4 if the tissue's quality is scored above or equal to 72. If the tissue's quality is scored below 60, do not continue further with agarose filling.

Note: If the tissue score is between 60 and 68, the agarose-filling and tissue slicing still might produce reasonable results, and a final decision for prolongation of the experiment has to be made case to case. However, lung tissue that did not meet the above-mentioned requirements, mostly fails in agarose filling.

4. Lung Tissue Inflation by Agarose Filling

4.1) Lift the tissue from the storage medium and drain excess media from the tissue. Transfer the lung tissue into the 15 cm culture dish prepared in 1.1.2.

4.2) Fill a 30 mL syringe with the low-melting point agarose from 1.1.3.

4.3) Prepare a peripheral venous catheter by removing the obturator and attach it to a 30 mL syringe

4.4) Identify a bronchus (0.5-3 mm in diameter) in the tissue that is ventilating an intact section of the tissue (see **Figure 2**).

4.5) Insert the cannula into the selected bronchus (0.5-3 mm diameter).

4.6) Gently push the cannula gently forward as far as possible.

4.7) Seal the bronchus around the cannula by compressing the bronchial wall around the cannula with forceps, ideally clamping any adjacent pulmonary artery at the same time.

4.8) Occlude other additional airways with a surgical clamp to prevent agarose leaking through these airways.

4.9) Lift the tissue with the forceps from the culture dish.

4.10) Manually pour the agarose with the syringe no faster than 0.3 mL/s. The speed of agarose filling might vary between approximately 0.05 and 0.3 mL/s due to the heterogeneous resistance of airways and/or atelectasis.

4.11) If high resistance while filling or agarose leaking from the tissue is observed, retry the whole procedure with a different bronchus from step 4.4. Perform troubleshooting as described below.

Note: The degree of agarose filling is highly dependent on the position of the catheter in the tissue and deep penetration of the catheter results in agarose filling of small cone like regions (*) of the lung tissue (**Figure 2C**).

4.11.1) In case of high resistance, try positioning of the catheter leads to proper filling of most regions of the tissue (#) (**Figure 2D**).

4.11.2) As plugs of early solidified agarose in the proximal bronchi or other airway obstructions (arrow) can lead to an incomplete filling of the tissue (**Figure 2E**), do not force agarose filling as this might lead to defects in the filled area, but not in a filling of the obstructed tissue parts.

4.11.3) If the respiratory tree derived from the cannulated bronchus is damaged during resection and the agarose filling results in a constant leaking of the liquid agarose (arrow in **Figure 2F**), insert the catheter into a more peripheral part of the airway system to fill at least a minor part of the tissue (*) (**Figure 2G**). Additionally, seal the damaged peripheral airway with a surgical clamp (arrow) (**Figure 2H**).

4.12) Apply agarose until the lung tissue is filled completely. Do not over-inflate the tissue as this may cause irreversible damage to the tissue structure and its cells.

4.13) Clamp the bronchus that was used for the filling immediately. Remove the cannula prior to clamping.

4.14) Incubate the tissue in the culture medium at 4 °C for 30 min to ensure agarose solidification.

4.15) If the resected tissue has multiple bronchial entries, repeat step 4.2 to 4.13 until all parts of tissue are filled with agarose.

4.16) Store the agarose filled lung tissue sections in 4 °C cold medium until slicing.

5. Precision-Cut Lung Slicing

5.1) Identify regions in the lung tissue that are solidly filled with the agarose. Solidly filled regions will not collapse when they are gently pressed with tweezers against the bottom of the cell culture dish.

5.1.1) Excise a 1-1.5 cm^3 block of regions described in 5.1, whereas one side still should be covered with pleura.

5.2) Attach each individual tissue block with the pleural side contacting the holder of the vibratome by using a cyanoacrylate glue.

Note: The pleura is slightly elastic and therefore impedes the cutting with the vibratome blade. Placed on the tissue holder, the pleura will not interfere with the cutting and, importantly, forms a natural barrier between the cyanoacrylate glue and the tissue's parenchyma allowing for minimal diffusion of the glue.

5.3) Slice the lung tissue with the vibratome with the following settings: thickness: 500 µm, frequency: 100 Hz, amplitude of the knife: 1.2 mm, forward speed of the blade of 3-12 µm/s, which depends on tissue stiffness. Reduce the protrusion-speed of the blade if the slice is not cut properly, or if the tissue block itself starts to vibrate.

5.4) Gently transfer the slice by lifting it with forceps from the vibratome tray into a well of a 12-well plate filled with cultivation medium. Finally, incubate the lung slices in an incubator under standard cell culture conditions.

5.5) Stop slicing when 2-3 mm of the tissue block are left unsliced since the cyanoacrylate glue may have compromised the tissue integrity of this region.

6. Generation of PCLS Punches

6.1) Transfer the lung slices from a single well to an empty 10 cm dish.

6.2) Place a 4 mm biopsy puncher orthogonally to the upper surface of a PCLS and start to move the puncher in clockwise and counterclockwise rotations.

6.3) Fill cell culture medium into the wells of a 96-well plate. Lift the tissue punches with forceps and transfer the punches into the wells of a 96-well plate. Finally, incubate the lung punches submerged in the medium prepared in 1.1.1. in a cell culture incubator under standard conditions (21% (v/v) oxygen, 5% (v/v) carbon dioxide and 95% humidity, at 37 °C).

7. Tissue Culture and Sample Harvesting

7.1) Culture the PCLS and punches overnight in an incubator under standard cell culture conditions.

7.2) Culture PCLS and punches under outlined condition for a maximum of 120 hours after their generation to ensure cellular viability and function.

7.3) For harvesting protein as well as RNA, wash PCLS and punches three times in phosphate buffered saline (PBS), transfer them into cryovials and snap-freeze in liquid nitrogen.

7.4) Sample medium supernatant of cultured PCLS punches for the analysis of secreted proteins.

7.3.1) For histological analyses, wash the PCLS and punches three times with PBS and fix them with 4% paraformaldehyde by incubating for 30 min at 37 °C. Finally, store the PCLS in PBS at 4 °C for further downstream staining.

REPRESENTATIVE RESULTS

PCLS generation

The generation of PCLS can be separated into four essential steps: surgical lung tissue resection, agarose filling, vibratome-based PCLS generation, and culture of PCLS. The resected lung tissue is filled with low-melting point agarose, which adds the required stiffness to the lung tissue for slicing and preserves the native lung structure and architecture. Of note, PCLS generation is highly time consuming, thus often overnight storage of the filled lung tissue in DMEM F-12 medium can be included as an additional step and PCLS generation is started on the next day. Depending on the following experimental setup, generated PCLS can be incubated overnight in standard cell culture medium containing 0.1% (w/v) fetal bovine serum, before experimental conditions are applied. 3D-LTCs were viable and exhibited cellular functionality (such as surfactant protein secretion) for up to 120 hours[22] in the culture conditions outlined in this protocol (**Figure 1**) and might be optimized upon further improvement thereof.

Agarose filling

For agarose filling of the tissue, a cannula of a peripheral venous catheter with a 1.3 mm diameter attached to the agarose-filled syringe was inserted into a bronchus at the surface of the cut tissue (**Figure 2A**). Bronchi are often localized near to a pulmonary artery. While the arteries have thinner walls and tend to collapse, bronchi exhibited a good visible lumen. Depending on the tissue's integrity, the catheter can be advanced through several generations of the respiratory

tree into the periphery of the lung. The penetrated bronchus was sealed around the cannula by using tweezers (**Figure 2B**). The pulmonary artery can be clamped with the tweezers at the same time. Afterwards, the tissue is lifted up and liquid agarose is gently instilled into the airways.

Depending on the position of the catheter, a majority of the tissue can be filled with liquid agarose (**Figure 2D**). Optionally, cone like parts of the lung tissue, which reflect the lung's parenchyma ventilated by the penetrated bronchus, might get filled with the agarose (**Figure 2C**). In both scenarios, a characteristic pattern of solidly filled tissue regions can be observed: first, a major part of the tissue is filled in wedges (**Figure 2D**), or secondly, smaller protruding round areas of thoroughly filled tissue regions appear (**Figure 2C**). If parts of the airways obstruct due to agarose clots or other causes, parts of the tissue might not be properly filled with agarose. Thus, only parts of the tissue might be applicable for slicing. In case of leakages during the agarose filling procedure, parts of the filled respiratory tree might get perforated and filling of the lung tissue gets nearly impossible However, possible workarounds include the filling via a more peripheral bronchus, a deeper penetration of the cannula into the distal airways (**Figure 2G**), or potential clamping of the leakage area (**Figure 2H**).

Precision-cut lung slicing

Tissue blocks at a length and width of 1-1.5 cm were excised from tissue regions, which were completely filled with solidified agarose **(Figures 3A-3B)**. Next, the individual tissue blocks were glued on the tissue holder of the vibratome **(Figure 3C)**. 500 µm thick PCLS were generated, whereas the tissue block at the vibratome was advancing forward with speeds between 3-12 µm/s. (**Figures 3D-3F**). Finally, the PCLS were submerged in cell culture medium containing 0.1% (w/v) fetal bovine serum and cultured at standard cell culture conditions, as outlined step 7.

Experimental readouts of human 3D-LTC **after 48h of culturing**

A representative immunofluorescence staining, as previously described by Alsafadi *et al.*[25], is shown in **Figures 4A-4C**. Immunolabeling of fibronectin (red) and cell nuclei (DAPI, blue), allowed for imaging of the preserved alveolar structure in the human 3D-LTC *ex vivo*. Treatment of the human PCLS punches with a profibrotic cytokine cocktail (including transforming growth factor beta 1, platelet derived growth factor AB, lipophosphatidyl acid, and tumor necrosis factor alpha) for 48 hours resulted in fibrosis-like changes in human 3D-LTCs. By qPCR, a significant induction of the fibrosis-relevant extracellular matrix components collagen type 1 and fibronectin genes in 3D-LTC punches was observed upon treatment with the profibrotic cocktail (**Figure 4D**). Additionally, protein levels of the mesenchymal marker vimentin were found upregulated in 3 out of 4 patients after treatment of 3D-LTC punches (**Figure 4E**).

Figure Legends

Figure 1: Workflow of PCLS generation

Tumor-free areas of lung resections are thoroughly inspected due to their tissue integrity. If the tissue is scored suitable for further use (scoring is explained in detail in the material and methods section), it is next filled with liquid agarose. Tissue blocks filled with solidified agarose are subsequently sliced with a vibratome. Submerged in cell culture medium, 3D-LTC are cultured up

to 120 h after their generation. Downstream analyses of the 3D-LTCs involve protein- or RNA-expression, live-tissue fluorescence imaging, as well as immunofluorescence staining after fixation of the tissue.

Figure 2: Filling the lung tissue with low-melting point agarose

The lung tissue is cannulated with a peripheral venous catheter which is inserted into a bronchus adjacent to the pulmonary artery (**Figure 2A**). Tweezers are used to fix the cannula in the bronchus and to clamp the pulmonary artery to avoid leaking of the liquid agarose. Liquid agarose at 42 °C is poured into the lung tissue with a 30 mL syringe (**Figure 2B**). A distal positioning of the cannula during filling will result in small areas of filled tissue **(Figure 2C)**, while proximal positioning will ensure the filling of a larger tissue volume **(Figure 2D)**. Any obstructions of the airways will reduce the amount of tissue volume that can be filled (**Figure 2E)**. In case of agarose leaking, a distal cannula positioning and/or clamping of the leakage side enables proper agarose filling of the lung tissue **(Figures 2F-2H)**.

Figure 3: Precision-cut lung slicing

A successfully agarose-filled lung tissue is used to excise a piece of a tissue block (1 cm x 1.5 cm x 1 cm) with a scalpel (**Figure 3A**). Next the excised tissue block is glued to the tissue holder, scale bar indicates 1 cm (**Figure 3B**). Preferably, the tissue is glued with its pleural surface to the surface of the tissue holder as shown in **Figure 3C**. 500 µm thick slices are cut by the vibratome with a sapphire knife in a 10°-15° angle relative to the tissue (**Figure 3D** and **3E**). The cutting procedure results in 2-3 cm^3 large intact lung slices, scale bar indicates 5 mm (**Figure 3F**). Additionally, by using a biopsy puncher, small reproducible punches with a diameter of 4 mm can be generated.

Figure 4: Experimental readouts of human 3D-LTCs after 48h of culture

A human 3D-LTC punch of 4 mm diameter was immunostained for fibronectin (in red) and DAPI (in blue) **(Figures 4A-4C**). Scale bars indicate 1000 µm. **Figure 4C** shows the merged image. RNA analysis of PCLS by quantitative RT-PCR shows significant increases of COL1A1 and FN1 gene expression by the profibrotic cocktail[25]. **Figure 4E** displays an immunoblot of whole protein lysates of PCLS, which were treated with a fibrotic cocktail[25]. Probing for Vimentin and β-Actin demonstrated an increased protein expression of the mesenchymal marker (vimentin) after treatment with profibrotic factors in patient samples 1, 3, and 4.

Table 1: Lung Agarose Filling Score

The Lung Agarose Filling Score (LAFS) correlates with the success-rate to fill a tissue resection with agarose for its subsequent vibratome-based PCLS production. The score sums up all points of criteria met by the tissue. An LAFS equal or above 72 predicts good agarose filling properties, a score below 60 predicts a highly probable failure of agarose filling of the tissue.

DISCUSSION

The protocol described in this manuscript covers the generation of PCLS from human lung tissue resectates by filling it with liquid agarose and subsequent vibratome slicing. Generation of tissue slices was demonstrated before for a couple of organs, like liver and brain, whereas the inherent

stiffness of these organs allowed direct slicing without any modification of the tissue. Of note, the initial proper preparation of the lung tissue is the most crucial step in generating PCLS. Agarose filling of the lung is the method of choice to stabilize its soft and elastic nature, and to ensure a homogenous and reproducible PCLS generation. Large airways of the resected lung tissue are cannulated to provide access to the small airways, as well as to the intact lung parenchyma. The lack of an intact pleura, which makes agarose filling nearly impossible, is a major reason why lung tissue is mostly not usable for lung slicing. Prospectively, a synthetic pleura originally designed to perform functional experiments on decellularized scaffolds could potentially be applied to achieve successful agarose filling of explants that lack an intact pleura[31]. Resections resulting in a human lung tissue piece with intact pleura are essential for generating tissue blocks for slicing. Resected tissue is more available due to tumor-free tissue from cancer resections than fully intact lobes or whole-lung explants of patients who underwent lung transplantation.

Commonly, two systems are used to produce PCLS: the Krumdieck tissue slicer[15] and vibratory microtomes (vibratomes). Tissue slicers generate slices by passing a tissue block through a metal vessel, which cuts the PCLS at 90° at the end of this vessel. Vibratomes generate PCLS by moving a vibrating knife horizontally over an anchored block of tissue that is submerged in a cooled medium bath, which compared to the Krumdieck slicer exerts less shear force on the tissue. This results in less harsh treatment of the tissue before cultivation. On the other hand, the vibratome cutting is more time and work consuming. In our hands, vibratome slicing enabled the production of a maximum of 100 PCLS or 500 PCLS punches in one day, sufficient for most experimental studies. PCLS can be cultured in various ways: (a) attached to Trans-wells, thus generating an air liquid interface (ALI) system, (b) as dynamic organ culture (DOC), or (c) submerged in cell culture medium at standard cell culture conditions. The in-detail cultivation of PCLS was previously described[22,23,25]; however, a common standard of cultivation conditions between their usage in various labs around the world is still missing. In particular, the culture time might be critical: as in murine PCLS, a loss of SFTPC positive alveolar type 2 cells is observed after 144 h, but not after 120 h[22]. In addition, metabolic activity seems to remain stable in murine[22] and human PCLS[25] for 120 h.

There are a couple of technical limitations for the generation of PCLS: the number and size of the resectates fluctuates over time; the efficiency of the agarose filling, which depends on the presence of intact pleura within the obtained tissue, determines the final success of the PCLS generation; and tissue destruction caused by pathological changes within the obtained (diseased) lung tissue might interfere with the PCLS preparation. Airway obstructions and fibrotic tissue lacking intact alveolar space impede with agarose filling and thus make slicing of the fibrotic tissue a demanding task. Emphysematous tissues as found in diseases such as COPD or alpha-1-anti-trypsin deficiency might not withstand the pressure of agarose filling, and will result in rupture of the alveoli and architectural artefacts. In these cases, the usage of low agarose concentration, *e.g.,* 1% (w/v), might be useful to decrease pressure and speed during agarose filling. Overall, the disease state of the tissue can dramatically limit the use of the tissue for PCLS generation. All these parameters determine the amount of PCLS that can be generated from lung

tissue, and also the amount of time it takes to produce the PCLS. Further limitations of PCLS are inconsistencies in between different lung slices with respect to size or tissue content, which requires further normalization steps for experiments. To overcome this, biopsy punches of similar regions of the same slice can be generated. This procedure is apt to reduce the tissue variability and, as an additional benefit, increase the number of PCLS samples that can be used for experiments.

In conclusion, human 3D lung tissue cultures from agarose filled PCLS provide a complex human model for studying lung physiology and diseases. The protocol provides a detailed description of the preparation of PCLS from resected lung tissue and their cultivation, and moreover addresses challenges in agarose filling of human lung resections and how to overcome them.

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DISCLOSURES

All authors declare no competing financial interests.

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Figure 1

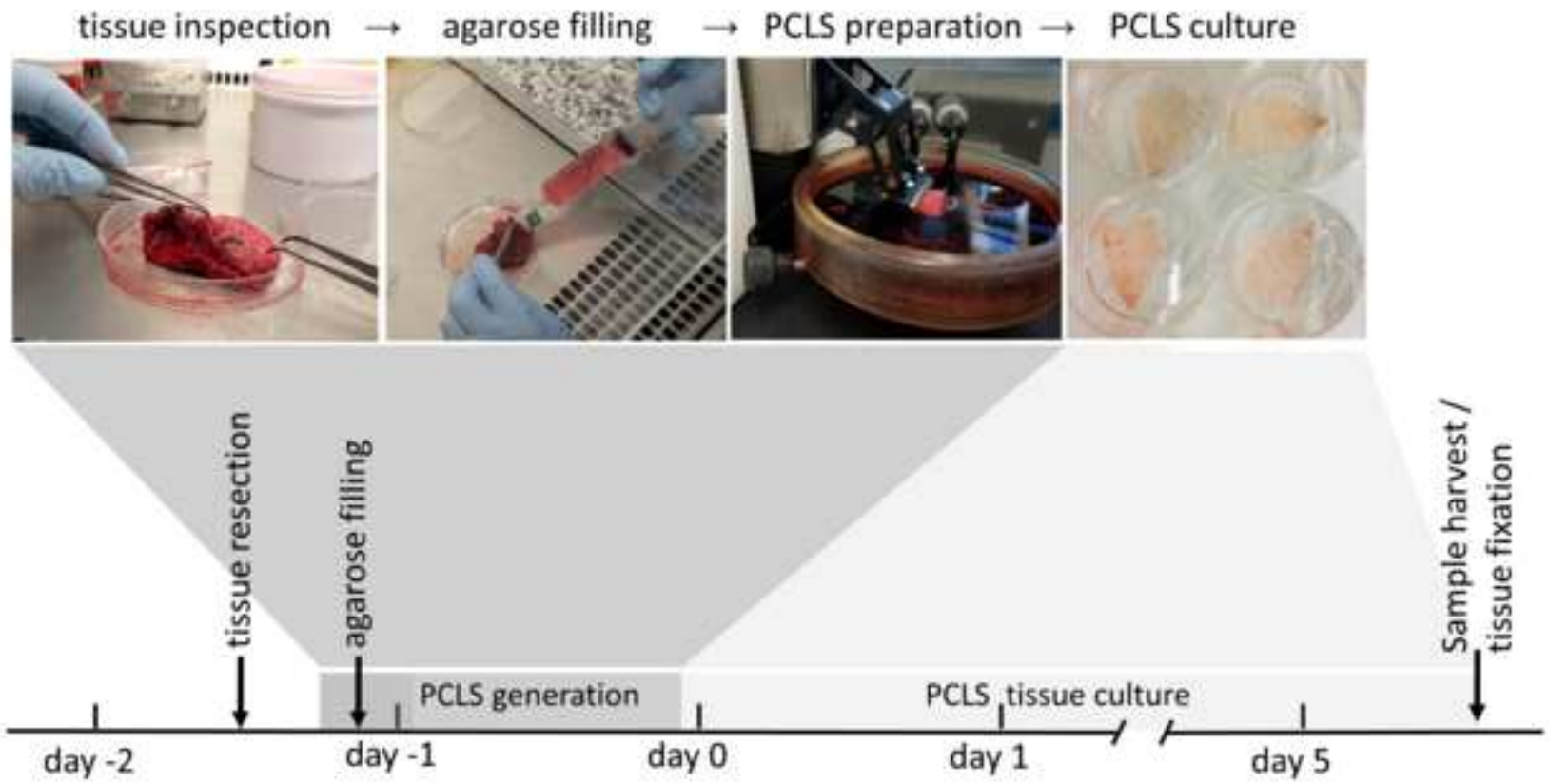

Figure 2

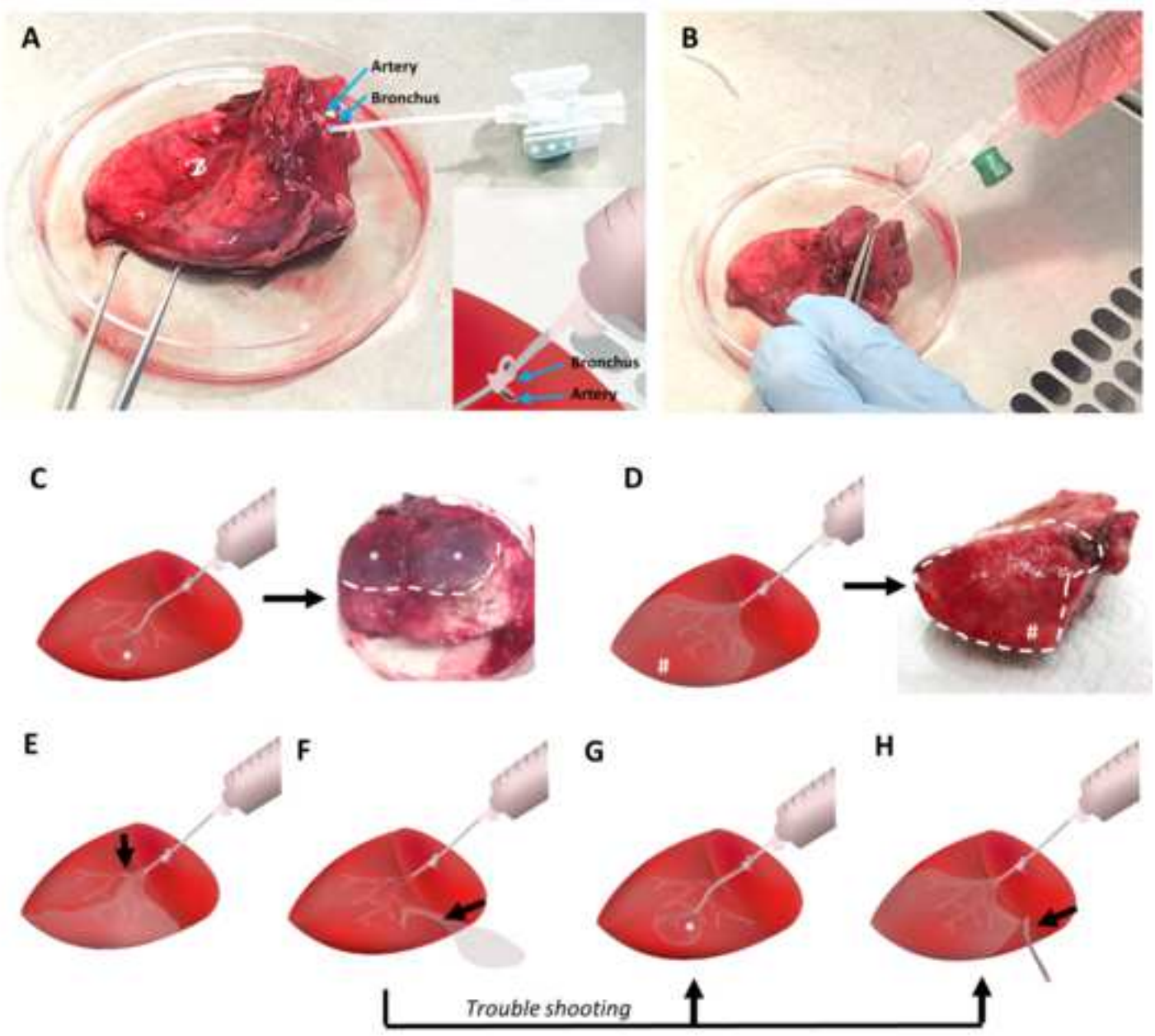

Figure 3

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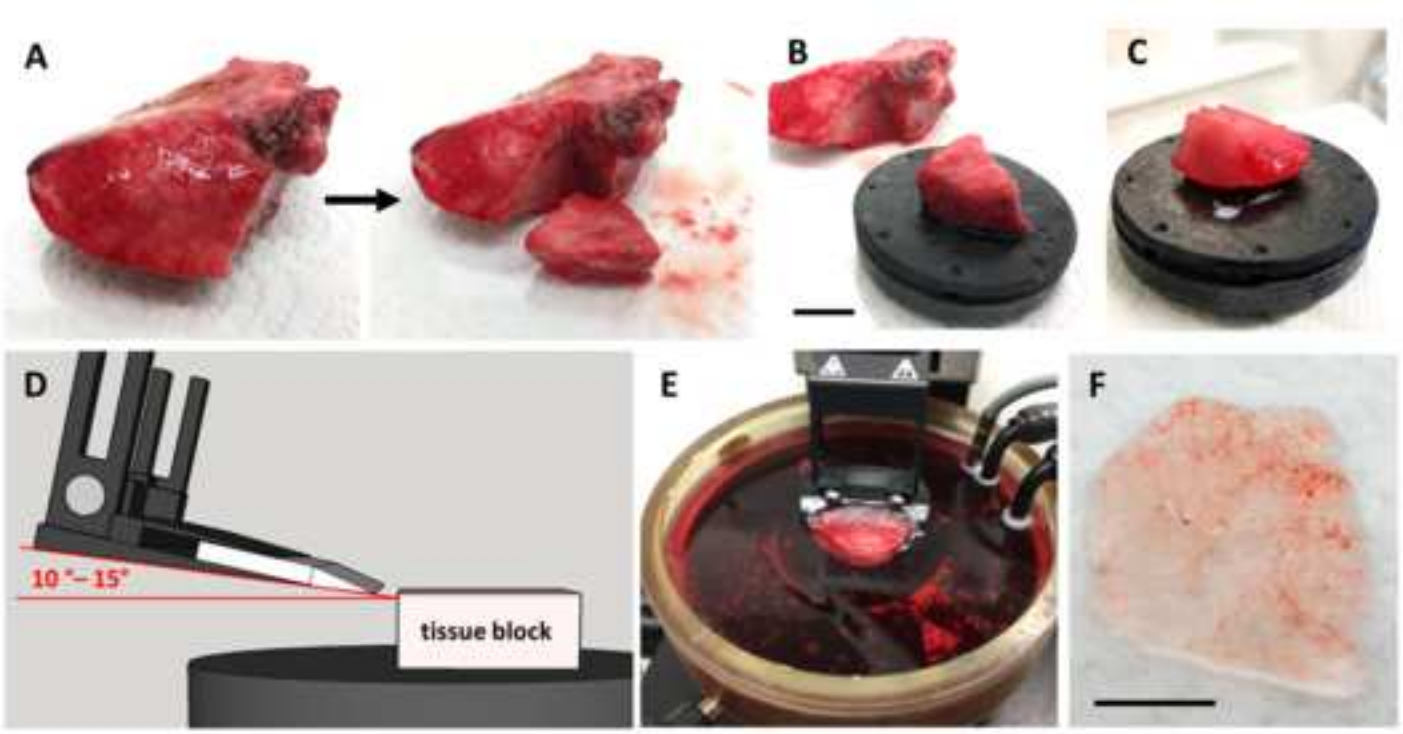

Figure 4

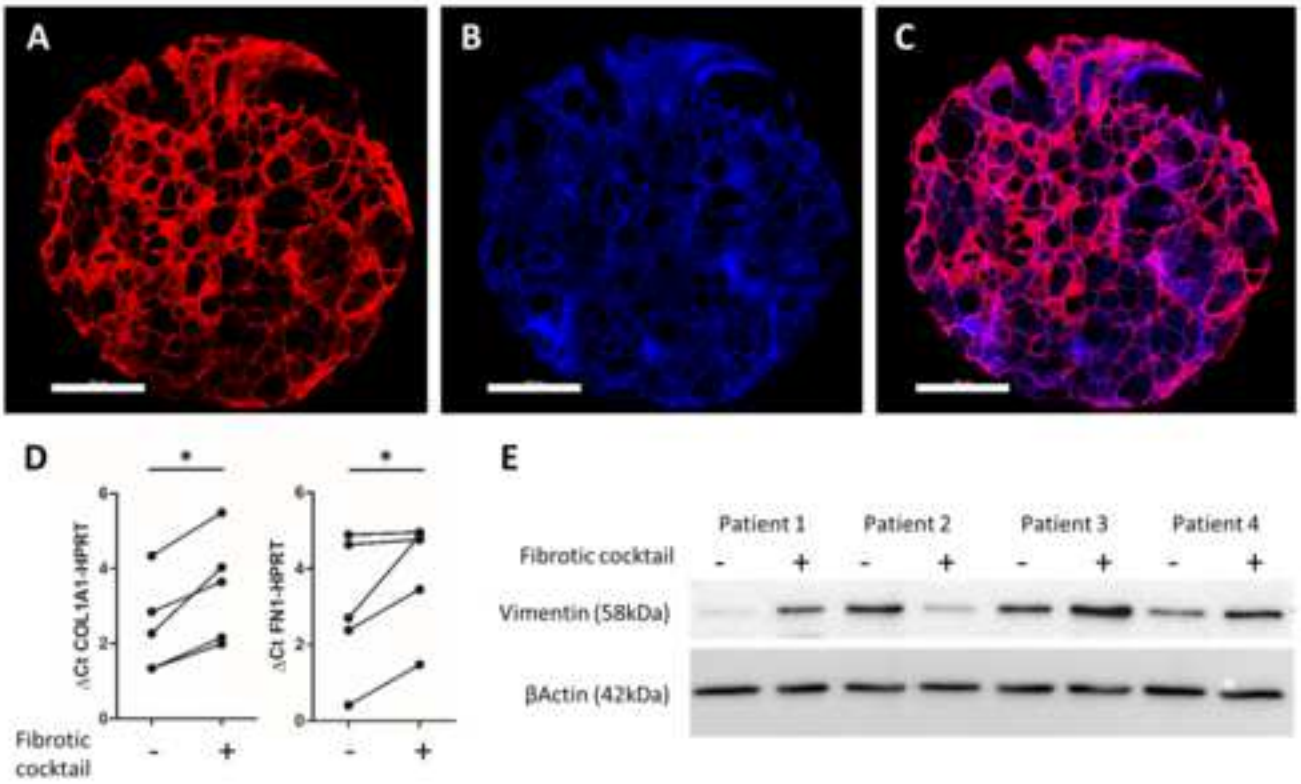

Table for the Lung Agarose Filling Score

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Criterion	Points
The tissue sample has intact pleural surface.	20
The tissue sample seems macroscopically intact, lacking incisions, squashing, ruptures and distortions.	20
The tissue sample contains at least one bronchus with a diameter >1mm.	20
The tissue sample contains no or only little amounts of blood.	4
The tissue sample was stored fully in medium and shows no obvious sign of atelectasis.	4
The tissue sample was resected within the last four hours.	4
The tissue sample is larger than 5cm in its largest diameter.	4
Score in sum:	

Table of Materials

Click here to download Table of Materials JoVE_Materials.xls

Name of Material/ Equipment	Company	Catalog Number	Comments/Description
Vibratome Hyrax V50	Zeiss	-	
Hyrax CU 65	Zeiss	-	
Vasofix Braunüle 18G	B. Braun Melsungen AG	4268130B	
30mL NORM-INJECT	Henke Sass Wolf	4830001000	
Guarded disposable scalpels, sterile	Swann-Morton		
Loctite 406	Henkel	LOCTITE 406	
Synthetic Single Crystal Sapphire	Delaware Diamond Knives	-	
Dulbecco's Modified Eagle Medium F-12 Nutreient Mixture (Ham) + L-Glutamine + 15mM HEPES	Gibco	31330-038	
Penicillin Streptomycin	Gibco by Life Technologies	15070-063	
Special process fetal bovine serum (Sera Plus)	Pan Biotech	P30-3702	
Disposable Biopsy Punch	pfm medical	48401	
96 Well, Black/Clear, Tissue Culture Treated Plate, Flat Bottom with Lid, sterile	Falcon / Corning	353219	
Agarose, low geling temperature	Sigma	A9414-100G	

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Author(s): GERCKENS, ALSAFADI, WAGNER, LINDNER, BURGSTALLER, KÖNIGSHOFF

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Institution: PULM DIVISION

Article Title: GENERATION OF HUMAN LUNG TISSUE CULTURES

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JoVE - JoVE58437

Generation of human lung tissue slices for disease modeling

Dear Dr. Bajaj,

Thank you very much for the expedite review of our submission JoVE58437 to the Journal of Visualized Experiment (JoVE). Attached, please find our revised manuscript entitled “Generation of human lung tissue slices for disease modeling". As required, all changes in the manuscript are marked in red.

We have substantially revised our original submission as suggested by the editor and reviewers of the initial submission, and responded to all editor and review comments. We thank the editor and the reviewers alike, especially for their time and valuable comments, all of which have significantly improved the quality of our initial manuscript.

Best regards,

Dr. Gerald Burgstaller.

Prof. Dr. Melanie Königshoff

Point by Point response to the editor's and reviewers' comments:

Editor:

1. Please take this opportunity to thoroughly proofread the manuscript to ensure that there are no spelling or grammar issues. The JoVE editor will not copy-edit your manuscript and any errors in the submitted revision may be present in the published version.

We proofread the manuscript and corrected typographical errors.

2. Please upload each Figure individually to your Editorial Manager account as a .png or a .tiff file. Please remove the titles and Figure Legends from the uploaded figures. The information provided in the Figure Legends after the Representative Results is sufficient.

We removed the title and figure legends, and provide the figures as .tiff files at 300 dpi.

3. Figures 4A-C: Please define the scale bar in the figure legend.

We added the following sentence to the figure legend: Scale bars indicate 1000 µm.

4. Figures 4D and 4E: Are they reprinted? If so, please obtain explicit copyright permission to reuse any figures from a previous publication. Explicit permission can be expressed in the form of a letter from the editor or a link to the editorial policy that allows re-prints. Please upload this information as a .doc or .docx file to your Editorial Manager account. The Figure must be cited appropriately in the Figure Legend, i.e. "This figure has been modified from [citation]."

The image was not used in previous publications. Alsafadi et al (2017), Am J Physiol Lung Cell Mol Physiol 312: L896–L902, shows different images acquired using the same technique.

5. Figure 4E: Please include a space between number and its corresponding unit (i.e., 58 kDa and 42 kDa).

We changed these units accordingly.

6. Please remove the embedded Table between lines 160 and 161 from the manuscript and upload the table separately to your Editorial Manager account in the form of an .xls or .xlsx file. Refer to the Table in step 3.2. Each table must be accompanied by a title and a description after the Representative Results of the manuscript text.

We separated the table into an xls-File. We provide the table legend in the manuscript. We corrected an error in 3.3.

7. Please number the figures in the sequence in which you refer to them in the manuscript text.

With the changes in the revised manuscript the figure numbers match their sequence of appearance in the text. We are convinced that the suggested order of figures is the most intuitive to the reader.

8. Please shorten the figure legend it possible. Details of the methodology should not be in the Figure Legends, but rather the Protocol.

We shortened the legend of Figure 2. And added the following methodology to step 4.1.11:

4.1.11) If high resistance while filling or agarose leaking from the tissue is observed, retry the whole procedure with a different bronchus from step 4.1.4. Troubleshooting can be performed as follows:

4.1.11.1) Note that the degree of agarose filling is highly dependent on the position of the catheter in the tissue and deep penetration of the catheter results in agarose filling of small cone like regions (*) of the lung tissue (**Figure 2C**). In case of high resistance, try positioning of the catheter leads to proper filling of most regions of the tissue (#) (**Figure 2D**)

4.1.11.2) As plugs of early solidified agarose in the proximal bronchi or other airway obstructions (arrow) can lead to an incomplete filling of the tissue (**Figure 2E**), do not force agarose filling, this might lead to defects in the filled area, but not in a filling of the obstructed tissue parts.

4.1.11.3) Note that if the respiratory tree derived from the cannulated bronchus is damaged during resection and the agarose filling results in a constant leaking of the liquid agarose (arrow in **Figure 2F**). Here, insert the catheter into a more peripheral part of the airway system to fill at

least a minor part of the tissue (*) (**Figure 2G**). Additionally, seal the damaged peripheral airway with a surgical clamp (arrow) (**Figure 2H**).

9. Please rephrase the Short Abstract to clearly describe the protocol and its applications in complete sentences between 10-50 words: “Here, we present a protocol to …”

We rephrased the short abstract accordingly.

10. Please rephrase the Long Abstract to more clearly state the goal of the protocol.

We rephrased the long abstract in order to clarify the goal of the protocol and to focus on the agarose filling of surgically resected tissue, as suggested by reviewer 3.

11. Please rephrase the Introduction to include a clear statement of the overall goal of this method.

We revised the introduction, as we did with the long abstract.

12. Please use SI abbreviations for all units: L, mL, µL, h, min, s, etc.

We changed all SI abbreviations accordingly. We believe that using the non SI units “degree” (angle) and “degree Celsius” (temperature) instead of the SI abbreviations radian (angle) and Kelvin (temperature) makes the protocol more accessible for the general reader.

13. Please include a space between all numbers and their corresponding units: 15 mL, 37 °C, 60 s; etc.

We formatted all units accordingly.

14. Please revise the protocol text to avoid the use of any personal pronouns (e.g., "we", "you", "our" etc.).

We changed this in the protocol part.

15. 3.1, 3.1.1, 3.2, 3.3, 4.1.13, 5.1.3, 5.1.6, 7.1.2, 7.2.1, and 7.3.2.: Please revise the protocol to contain only action items that direct the reader to do something. The actions should be described in the imperative tense in complete sentences wherever possible. Avoid usage of phrases such as “could be,” “should be,” and “would be” throughout the Protocol. Any text that cannot be written in the imperative tense may be added as a “Note.”

We changed these expressions accordingly.

16. 6.1.3: Please specify the cell culture medium used and the culture conditions.

Culture medium has been specified in 1.1.1. To be even more clear, we referenced this step in 6.1.3.

17. 7.3.2: Please spell out PFA.

We changed step 7.3.1. and 7.3.2 to:

7.3.1) For histological analyses, wash the PCLS and punches three times with PBS and fix them with 4% paraformaldehyde by incubating for 30 min at 37 °C. Finally, store the PCLS in PBS at 4 °C for further downstream staining.

18. Please include single-line spaces between all paragraphs, headings, steps, etc.

We changed this accordingly.

19. There is a 2.75 page limit for filmable content. Please highlight 2.75 pages or less of the Protocol (including headings and spacing) that identifies the essential steps of the protocol for the video, i.e., the steps that should be visualized to tell the most cohesive story of the Protocol. Remember that non-highlighted Protocol steps will remain in the manuscript, and therefore will still be available to the reader.

We highlighted (yellow) 2.5 pages of the protocol from page 6 to 8.

20. Please ensure that the highlighted steps form a cohesive narrative with a logical flow from one highlighted step to the next. Please highlight complete sentences (not parts of sentences). Please ensure that the highlighted part of the step includes at least one action that is written in imperative tense.

We modified the protocol, so the highlighted text (yellow) contains actionable items in imperative tense only.

21. Please discuss all figures in the Representative Results. However, for figures showing the experimental set-up, please reference them in the Protocol.

We referenced figure 1-3 in the protocol.

22. References: Please do not abbreviate journal titles.

We used the JoVE Citation Style for Endnote from the JoVE website.

Reviewer #1:

Manuscript Summary:

This is a well-written protocol designed to allow researchers to generate precision cut lung slices from diseased lung resectates. The authors provide documentation of the steps from receipt to the start of experiments with this tissue. Importantly, the generation of these slices provides a substantial advantage to human lung research as the tissue is generated from the correct host in the correct site of disease. The applications are numerous as documented in the introduction. This reviewer does have a few questions for the authors.

We thank the reviewer for the valuable and constructive comments to our manuscript. Within the revised version, we addressed the questions of the reviewer, which we feel resulted in an improved manuscript.

Major Concerns:

1. The authors mention cold ischemia as, a problem for generation of slices; however, they do not mention reperfusion injury. Are there any specific interventions that one should consider regarding reperfusion injury (glutathione?)?

Thank you very much for raising this important point. We agree that storage and ischemia time remain crucial in ex-vivo culture preparation. The tissue storage is based onhypothermic organ storage, which is decreasing the metabolic rate and energy requirement of the tissue, which altogether is focused on donor lung preservation[1]. However, our model clearly lacks vascular reperfusion. Therefore, we cannot relate tissue injury to the clinical reperfusion of lung injury.

2. Have the authors attempted to improve cold ischemia by slightly increasing storage temps? Is there a better storage temp than 4 degrees?

We did not attempt to do so yet due to limited amount of tissue available, however, we agree that this would be an important step to consider for future studies.

3. The authors mention inspection and selection criteria prior to making PCLS; however, does this

correlate with alive tissue once the slices are made? Are there other criteria that could be considered when thinking about whether the tissue is viable (i.e., ciliary motility, contractility of airways)?

The tissue quality scoring is an attempt to apply objective criteria to accept or reject tissue for agarose filling. These criteria have derived from our accumulated experience over the years. Tissue that fail in agarose filling might otherwise be used for human tissue sampling and experiments, therefore it is an ethical imperative to us, to predict tissue performance in agarose filling. At this stage, the score is not suitable to be directly predictive for tissue viability. We have assessed tissue culture viability extensively in our system using metabolic assays (such as MTT-Assay, Calcein-Staining) as well as functional readouts, such as cilia movement and surfactant secretion [2].

4. The authors mention that slices are only viable for 120hrs post-creation. Is this true? This reviewer has heard of other labs that are able to keep slices in culture for months.

We have observed consistent viability of cultured PCLS for up to 120h post-creation[2], however, we did not intend to imply that there is a general time limit for culture as the choice of media and media supplements might extend or alter persistence of the tissue's native cell composition and viability[2]. We have adapted our manuscript accordingly.

5. Do the authors find problems with contamination with fungus or yeast? If so, can they comment on treatment options and how they decide which slices get specific antibiotic/antimycotic interventions?

The tissue is obtained surgically sterile and lung tissue with known bacterial and mycotic infections, such as pseudomonas, fungi etc is not used for PCLS. However, to further prevent potential contamination, the culture media is supplemented with penicillin and the broad-spectrum antibiotic streptomycin, as well as an antimycotic Amphotericin B as outlined in the manuscript (XX). In our hand bacterial or mycotic contamination did not occur.

6. How long before experiments is FBS removed from the slices?

The PCLS are continuously cultured in 0.1% FBS. FBS is removed for protein/RNA extraction by washing of the PCLS prior to snap-freezing.

Minor Concerns:

Please consider citing the following articles:

Cooper, P. R., Lamb, R., Day, N. D., Branigan, P. J., Kajekar, R., San Mateo, L., … Panettieri, R. A. (2009). TLR3 activation stimulates cytokine secretion without altering agonist-induced human small airway contraction or relaxation. American Journal of Physiology-Lung Cellular and Molecular Physiology, 297(3), L530-L537.

Kennedy, J. L., Koziol-White, C. J., Jeffus, S., Rettiganti, M. R., Fisher, P., Kurten, M., … Kurten, R. C. (2018). Effects of rhinovirus 39 infection on airway hyperresponsiveness to carbachol in human airways precision cut lung slices. The Journal of Allergy and Clinical Immunology.

An SS, Wang WC, Koziol-White CJ, Ahn K, Lee DY, Kurten RC, et al. TAS2R activation promotes airway smooth muscle relaxation despite beta(2)-adrenergic receptor tachyphylaxis. Am J Physiol Lung Cell Mol Physiol 2012; 303:L304-11

Cooper PR, Kurten RC, Zhang J, Nicholls DJ, Dainty IA, Panettieri RA. Formoterol and salmeterol induce a similar degree of beta2-adrenoceptor tolerance in human small airways but via different mechanisms. Br J Pharmacol 2011; 163:521-32.

We thank the reviewer for bringing these interesting articles to our attention and have included the studies by Kennedy et al (2018) and Cooper et al (2011) to highlight the state-of-the-art work that has been performed in human PCLS with respect to modulation of human airway function.

Reviewer #2:

Manuscript Summary:

The manuscript „Generation of human lung tissue slices for disease modeling by Gerckens et al. Deals with the preparation of human PCLS as versatile tool for various purposes. The use of human ex vivo tissues allows much better extrapolation to the human in vivo situation than the use animal tissue. Furthermore, this method successfully replaces animal experiments and is in line with current EU directives. The methods are thoroughly described and the methods for tissue slicing i.e. Krumdieck Slicer and vibratome are well balanced. Analysis of RNA, immunofluorescence and the use of fibrotic cocktails allow a plethora of application. It was a pleasure to read the manuscript and I can recommend publication without any changes.

We thank the reviewer for the positive feedback

Reviewer #3:

Manuscript Summary:

The work by Gerckens et al is an important contribution to lung biology. Specifically, this work contributes to emerging knowledge regarding preparation, storage, and utility of human lung slices. The work should therefore be well received and highly cited. However, the general techniques of preparing human lung slices from donated lung have been previously published by multiple groups; the authors should make this clear and avoid the repetitive description, such as slicing the lung tissue. Instead, the distinguishable advantage of the presented technique should be emphasized, which is to prepare slices from surgically resected lung sample so that the accessibility to diseased lung is significantly increased. On the same token, the challenge to implementing the technique should also be clearly pointed out, i.e. to retain the agarose during the lung inflation when the pleural membrane was not intact any more.

We thank the reviewer for the insightful and thoughtful comments to our manuscript. We agree with the important points to clarify the unique focus of this protocol and have adapted our manuscript accordingly.

Major Concerns:

1. Ideally, the lung tissue samples should have an intact pleural surface or surgically sutured pleural membrane around the tissue except the resection surface, otherwise it is extremely hard to maintain the sealing during inflation. It is hard to interpret "intact pleural surface at a least two sides of the tissue" in the manuscript (how many sides are we expecting here?).

This is a good point, the wording we used was misleading. We clarified that we refer to an intact pleural surface of the tissue piece. We changed the sentence in the Lung Agarose Filling Score accordingly.

2. There is also confusion regarding the filling process as it pertains to multiple bronchial openings on the section surface. Our personal experience is to cannulate all of the big openings, instead of doing one by one, for 2 reasons: 1) leaking can be from any of the airway openings and there is no way you will know that before injecting agarose; 2) Once the tissue is partially inflated, the tissue distortion and the concern of agarose leakage from the inflated part make very difficult to cannulate some other airways to inflate the lung. Can the authors please clarify.

We agree that the occlusion of the other airways in the tissue is beneficial to prevent agarose leaking, possible through collateral ventilation within the tissue. However, in our experience the cannulation

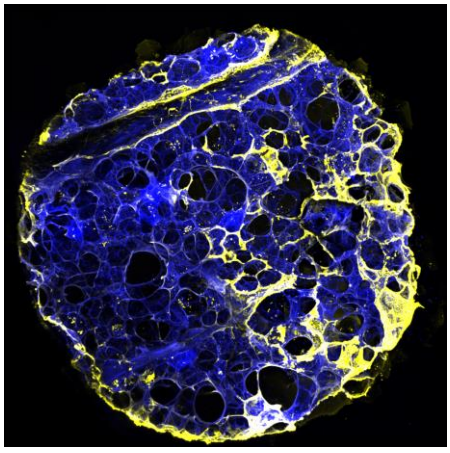
of a second or third airway after filling of the first airway does not represent a problem. In small tissue parts however the cannulation of several airways in the first place can hamper the overview over the tissue and tissue manipulation. Therefore, we added a step to the protocol recommending the occlusion of the other airways without prior cannulation.

4.1.7) Seal the bronchus around the cannula by compressing the bronchial wall around the cannula with a forceps, ideally clamping any adjacent pulmonary artery at the same time.

4.1.8) Occlude other additional airways with a surgical clamp to prevent agarose leaking through these airways.

3. The authors should show the slices that contain the airways, not only because lung slices are usually used to study airways diseases, but also because it reflects the quality of a good preparation.

We agree that airways are an important anatomical structure studied in PCLS, however the manuscript focuses on studies of lung alveolar parenchyma. We have previously published micrographs of small airway containing PCLS in a cited publication[3]. Here is an example from this research article:



This micrograph shows a PCLS punch after 120h with Collagen type 1 immunofluorescence staining (yellow), DAPI nuclei staining (blue). In the upper quarter of the punch, an airway is visible.

4. It is noted that 3% agarose is used in preparing the slices. This concentration is quite high which make it easy to solidify when temperature dropped and may cause problem while filling small airways. The authors should also consider using lower concentration when inflating COPD lung sample. Furthermore, lower concentrations will enable better characterization of airway narrowing studies.

We thank the reviewer for this valuable suggestion and agree that titration of agarose concentration is an important issue. Low concentrations of agarose will prolong the solidification process, lower the melting temperature of agarose and result in less rigid agarose filled tissue, as higher concentrations result in the opposite. In our experience obstruction of airways by early solidified agarose clots occurs in very large tissue parts only. As the reviewer noted, we describe filling of smaller tissue parts, here we found 3% (w/v) agarose concentration optimal. In personal communication with other research groups in the field, we found consistently that higher concentrations of agarose clearly facilitated slicing with the vibratome, however, we agree that lower concentration could be beneficial for agarose filling of emphysematous lungs.

We added the following sentence to the discussion:

Emphysematous tissues as found in diseases such as COPD or alpha-anti-trypsin deficiency might not withstand the pressure of agarose filling, and thus will result in rupture of the alveoli and tissue architecture artefacts. Here, the usage of a lower agarose concentration, e.g. 1% (w/v) might prevent this problem by decreasing pressure and speed during agarose filling.

Reviewer #4:

Minor Concerns:

2.1: If the tissue is stored at 4°C and submersed in ice-cold medium, the question arises whether this would impair the filling process with the agarose solution by causing premature polymerization of the polymer within the cooled tissue. Additionally to this: Is the excess media that might still reside within the tissue after submerse storage drained? If not, isn't this risking additional dilution of the introduced agarose solution by an unknown dilution factor (albeit potentially small) just like excess blood would as depicted in the inclusion criteria for tissue?

We thank the reviewer for bringing up this important point. Excess media drains spontaneously from the tissue when lifting it from the medium and placing it on the cell culture dish. To clarify this for readers we added the following sentence to the protocol:

4.1.1) Lift the tissue from the storage medium and drain excess media from the tissue. Transfer the lung tissue into the 15 cm culture dish prepared in 1.1.2.

The problem of premature agarose solidification in the 4°C cold tissue seems plausible, however our experience shows that filling at an appropriate pace prevents this problem. The excess media is not explicitly drained to avoid tissue trauma (squeezing, additional atelectasis), however when taking the tissue out of the media, minor amounts of media drain spontaneously from the airways. Excess of blood in our opinion is less a problem due to dilution of the agarose solution but rather a sign of tissue trauma during surgery and the introduction of tissue hematoma. Tissue hematoma compromise tissue and pleural integrity and are therefore a predictor of "bad" agarose filling performance.

4.1.9: As agarose introduction is described in great detail, is it controlled manually or through a pump? How is the speed of agarose introduction controlled?

This is also a very important point, which we addressed in the revised version. Agarose application is controlled by hand. While a pump would be ideal for an automated process, we strongly feel that manual filling of the tissue allows for a better assessment of each individual tissue section. This is of particular importance as human tissue from lung resection exhibits a high degree of heterogeneity and thus needs adaptation as outlined in this protocol. For example, if very low resistance is observed when filling with the syringe, this is a strong hint for a leak in the tissue. High resistance could be a

sign for wrong anatomical localization of the cannula. To clarify this is the protocol we changes step 4.1.10:

4.1.10) Manually pour the agarose with the syringe not faster than 0.3 mL/s. (Note: speed of agarose filling might vary between approximately 0.05 and 0.3 mL/s due to the heterogeneous resistance of airways and/or atelectasis.

5.1.3: Has the degree of diffusion of the glue been checked? Are there changes in viability or responsiveness to treatments when comparing more "central" and more "subpleural" slices generated from a single tissue block despite discarding the last 2-3 mm of the tissue as described in 5.1.6?

We are using cyanoacrylate-based glues that are commonly used in surgery and have not systematically analyzed potential diffusion. Cyanoacrylate polymerizes within seconds and the polymerized cyanoacrylate becomes inert.

PCLS cultivation: Has there been any signs of fibroblast outgrowth/differentiation due to usage of (albeit minor) concentrations of serum in the culture medium?

This is an important point. Fibroblast outgrowth is certainly a concern, when using serum concentrations in ex-vivo culture and we have observed an increase in fibroblasts when using higher concentrations of FBS and this is much reduced in our current protocol, in which we use 0.1% FBS to still support cell survival[2], but prevent significant fibroblast outgrowth and overgrowth.

Literature:

1 de Perrot, M., Liu, M., Waddell, T. K. & Keshavjee, S. Ischemia-reperfusion-induced lung injury. *Am J Respir Crit Care Med.* **167** (4), 490-511, (2003).

2 Uhl, F. E. *et al.* Preclinical validation and imaging of Wnt-induced repair in human 3D lung tissue cultures. *Eur Respir J.* **46** (4), 1150-1166, (2015).

3 Alsafadi, H. N. et al. An ex vivo model to induce early fibrosis-like changes in human precision-cut lung slices. Am J Physiol Lung Cell Mol Physiol. 312 (6), L896-L902, (2017).